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Special Report

International Society for Analytical Cytology Biosafety Standard for Sorting of Unfixed Cells

Ingrid Schmid,[1]* Claude Lambert,[2] David Ambrozak,[3] Gerald E. Marti,[4] Delynn M. Moss,[5] and Stephen P. Perfetto[3]

[1]Department of Hematology/Oncology, David Geffen School of Medicine at UCLA, Los Angeles, California
[2]Immunology Laboratory, University Hospital St. Etienne, St. Etienne, France
[3]Vaccine Research Center, NIAID, National Institute of Health, Bethesda, Maryland
[4]Flow and Image Cytometry Section, Division of Cell and Gene Therapies, Laboratory of Medical and Molecular Genetics, CBER FDA, National Institute of Health, Bethesda, Maryland
[5]Centers for Disease Control and Prevention, Atlanta, Georgia

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Background: Cell sorting of viable biological specimens has become very prevalent in laboratories involved in basic and clinical research. As these samples can contain infectious agents, precautions to protect instrument operators and the environment from hazards arising from the use of sorters are paramount. To this end the International Society of Analytical Cytology (ISAC) took a lead in establishing biosafety guidelines for sorting of unfixed cells (Schmid et al., Cytometry 1997;28:99–117). During the time period these recommendations have been available, they have become recognized worldwide as the standard practices and safety precautions for laboratories performing viable cell sorting experiments. However, the field of cytometry has progressed since 1997, and the document requires an update.

Methods: Initially, suggestions about the document format and content were discussed among members of the ISAC Biosafety Committee and were incorporated into a draft version that was sent to all committee members for review. Comments were collected, carefully considered, and incorporated as appropriate into a draft document that was posted on the ISAC web site to invite comments from the flow cytometry community at large. The revised document was then submitted to ISAC Council for review. Simultaneously, further comments were sought from newly-appointed ISAC Biosafety committee members.

Results: This safety standard for performing viable cell sorting experiments was recently generated. The document contains background information on the biohazard potential of sorting and the hazard classification of infectious agents as well as recommendations on (1) sample handling, (2) operator training and personal protection, (3) laboratory design, (4) cell sorter set-up, maintenance, and decontamination, and (5) testing the instrument for the efficiency of aerosol containment.

Conclusions: This standard constitutes an updated and expanded revision of the 1997 biosafety guideline document. It is intended to provide laboratories involved in cell sorting with safety practices that take into account the enhanced hazard potential of high-speed sorting. Most importantly, it states that droplet-based sorting of infectious or hazardous biological material requires a higher level of containment than the one recommended for the risk group classification of the pathogen. The document also provides information on safety features of novel instrumentation, new options for personal protective equipment, and recently developed methods for testing the efficiency of aerosol containment. Published 2007 Wiley-Liss, Inc.†

Key terms: flow cytometry; occupational health; biohazards; cell sorting; biosafety; aerosol containment

INTRODUCTION

In 1994 the International Society of Analytical Cytology (ISAC), an association representing researchers involved

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*Correspondence to: Ingrid Schmid, Department of Hematology/Oncology, David Geffen School of Medicine at UCLA, 12-236 Factor Building, Los Angeles, CA 90095, USA.

E-mail: schmid@mednet.ucla.edu

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in cytometry, recognized the need to formulate safety guidelines for sorting and analysis of unfixed cells to provide laboratories with recommendations for practices to reduce the potential for biohazard exposure of instrument operators. To this end ISAC initiated the formation of a Biohazard Working Group charged with this task. After extensive discussion and review, ISAC adopted the document as the official guidelines, and they were published in 1997 (1). Many concepts addressed in the 1997 document are valid today, however, the field of cytometry and safety practices have progressed since 1997.

- Advances in cell sorter technology made high-speed cell sorting more prevalent and changed the biohazard potential of cell sorting experiments.
- New and less expensive options for personal protection of operators are available.
- Instrument manufacturers responded to the need for improved operator protection, and they have introduced instrumentation containing novel safety features.
- Newly designed safety attachments for cell sorters are commercially available.
- With the availability of compact sorters and their ease of operation many more laboratories have incorporated cell sorting into their experimentation, but often do not have dedicated operators to perform cell sorting experiments.
- Simpler, bead-based techniques for measuring the efficiency of aerosol containment during cell sorting have been developed.
- Advances in cell biology have increased the need for live infectious cell sorting for cell culture and experiments involving molecular genetics.
- There is a desire to use flow sorted cells clinically either for ex vivo expansion or for direct infusion into patients.

Hence, the previous published guidelines are outdated, and the generation of a new standard has become a pressing issue for the flow cytometry community worldwide, particularly as more and more laboratories conduct cell sorting experiments involving samples with variable and sometimes complex levels of biohazard potential such as genetically engineered cell preparations. The current ISAC Biosafety Committee has been charged with generating an updated standard that reflects the present knowledge and occupational safety practices.

Purpose

The purpose of this document is to provide a written standard that modifies or expands the 1997 guideline document for handling and sorting of potentially biohazardous specimens and includes methods to assess the risk of exposure of laboratory personnel to biological and/or toxic aerosols that may be produced by deflected-droplet fluorescence-activated cell sorters. The possibility exists that sorter operators could become infected with biological agents contained in the specimens they are sorting (2–5). Cell sorter operators are also at risk of exposure to laser radiation; therefore the document has been expanded to include recommendations pertaining to the use of lasers on cell sorters. Laboratories following the standard practices outlined here aid in preventing exposures of laboratory personnel to infectious agents that are present in unfixed cells. Recommendations in this document focus on cell sorting of live, unfixed samples. However, it is important to note that functional measurements on cells, e.g., evaluation of calcium flux or membrane potential, certain apoptosis assays, cytokine assays, or live DNA or RNA staining, preclude cell fixation, and when performed on jet-in-air flow cytometers, can expose operators or others involved in these experiments to potentially hazardous aerosols and sample splashes. Therefore, the standard applies to all procedures whenever unfixed samples are run through a jet-in-air flow cytometer or a sorter that combines a flow cell with jet-in-air sorting.

Biohazard potential of unfixed cells. Typical biological specimens that are subjected to cell sorting include (but are not limited to) peripheral blood leukocytes, bone marrow, splenocytes, thymocytes, sperm cells, cells from primary and immortalized cultures from humans, nonhuman primates, other species, and transgenic animals. These samples can harbor known and unknown infectious agents, such as hepatitis viruses (A, B, C, D[delta]), human immunodeficiency viruses (HIV-1, -2), or cytomegalovirus. Hepatitis B, C, D, and HIV viruses have been classified as carcinogenic for humans by the International Agency for Research on Cancer as have other viruses that are encountered in biological specimens, e.g., Epstein Barr virus, human T-lymphotropic viruses, Kaposi sarcoma herpesvirus/human herpesvirus 8, Herpesvirus saimiri, and simian virus 40 (6–9). Samples may contain novel viral or bacterial gene transfer vectors containing foreign genes or genes of interest. The vector may deposit the foreign genes of interest into the cells, with the vector or portions of the vector, either extracellularly or the genes may integrate into the host cell genomic DNA. The foreign genes may or may not be expressed in the host cells and may also contain genomic sequences of potentially infectious organisms or sequences of unknown function that could exhibit toxic or oncogenic effects. Occupational transmissions across species to humans of retroviral agents such as simian type D retrovirus are of particular concern as these animal viruses could be introduced into the human population by this route (10). Most known pathogens encountered when sorting clinical or research samples are transmitted by the percutaneous route, by direct exposure of broken skin or mucous membranes, or by ingestion. Some may be transmitted by inhalation of organism-containing droplets (Table 1) that are generated either through laboratory manipulations (11) or the sorting process (12). Although HIV viruses and Hepatitis viruses are primarily transmitted through the percutaneous route into unhealed or broken tissues, infection through aerosolization of viral particles has been documented for Hepatitis B (13) but not for HIV. However, transmission of the HIV virus has been described through ingestion of HIV-infected breast milk (14). Therefore, HIV can potentially infect an individual via the oral mucosal route. Further, inhalation of droplets from samples containing other pathogenic organisms, such as enteric protozoans or bacteria, are possible by the naso-oral route. Samples may not

Table 1
Infectious Agents Associated with Laboratory-Acquired Infections due to Manipulation with Biological Samples

Agent	Source of infection	Species	Route of infection	Biosafety level: Practices, safety equipment, and facilities
Hepatitis B, C, D virus	Blood, cerebrospinal fluid, urine, tissues	Human, naturally or experimentally infected primates	Inoculation, exposure of mucosal membranes to aerosols, broken skin	BSL2, BSL3 in case of aerosol production, large quantities or high concentrations
Herpes virus simiae (B virus)	Primary cultures (Rh monkey kidney cells)	Macaque or human	Inoculation, aerosol inhalation minimal	BSL3, BSL4 for large quantities or high concentrations
Herpes simplex 1,2 varicella virus	Ubiquitous	Opportunistic pathogen in immunocompromised host	Direct contact or aerosol inhalation	BSL2
Cytomegalovirus; Epstein–Barr virus (EBV)	Blood, tissues, EBV-transformed cell lines	Human	Inoculation, exposure of mucosal membranes to aerosols, broken skin	BSL2
Herpes 6,7 virus	Blood, bronchoalveolar lavage	Human	Risk not known	BSL2
Influenza virus	Bronchoalveolar lavage, respiratory tissues	Human, naturally or experimentally infected animals	Aerosol inhalation	BSL2
Lymphocytic chorio-meningitis virus	Blood, cell cultures, nasopharynx secretions, bronchoalveolar lavage, tissues	Nude mice, SCID mice, naturally infected macaques or marmosets, possibly man	Inoculation, exposure of mucosal membranes to aerosols, broken skin, well documented transmission by aerosol inhalation	BSL2, BSL3 in case of aerosol production, large quantities or high concentrations
Poliovirus	Fluids, tissues, respiratory secretions	Naturally or experimentally infected nonhuman primates, transgenic mice	Ingestion, parenteral inoculation	BSL2 practices by vaccinated personnel, use WHO guidelines for establishing BSL2/polio and BSL3/polio laboratories after wild polio has disappeared, when oral vaccination has stopped BSL4 for work with wild polio
Pox viruses; genetically engineered recombinant vaccinia virus	Lesion fluid, tissues, respiratory secretions, bronchoalveolar lavage	Infected volunteers or animals	Inoculation, exposure of mucosal membranes to aerosols, broken skin	BSL2 practices by vaccinated personnel
Human immunodeficiency virus (HIV-1, 2) Retroviral vectors containing full-length infectious genomes	Blood, body fluids, tissues	Human	Inoculation, exposure of mucosal membranes to aerosols (containing concentrated virus), broken skin	BSL2, BSL3 in case of aerosol production, large quantities or high concentrations
Simian immunodeficiency virus (SIV)	Blood, body fluids, tissues	Macaque	Inoculation	BSL2, BSL3 in case of aerosol production, large quantities or high concentrations
Vesicular stomatitis virus	Blood, body fluids, tissues	Infected animals, humans	Exposure to infectious droplets or aerosols, direct skin and mucosal membrane exposure	BSL3, BSL2 for laboratory-adapted strains with demonstrated low virulence

Table 1
Infectious Agents Associated with Laboratory-Acquired Infections due to Manipulation with Biological Samples (continued)

Agent	Source of infection	Species	Route of infection	Biosafety level: Practices, safety equipment, and facilities
HTLV-1, 2 virus	Blood, body fluids, tissues	Human	Inoculation, exposure of mucosal membranes to aerosols (containing concentrated virus), broken skin	BSL2, BSL3 in case of aerosol production, large quantities or high concentrations
Coxiella burnetii	Blood, urine, tissues	Infected animals, humans	Inoculation, exposure to infectious aerosols	BSL2, BSL3 for cell culture and manipulation of tissues
Rickettsia prowazekii	Infected tissues	Naturally infected, nonhuman primates	Inoculation, aerosol inhalation	BSL2, BSL3 for tissue cultures of infected cells
Brucella	Blood, cerebrospinal fluid, tissues	Human, experimentally infected animals, sheep	Inoculation, direct skin contact	BSL2, BSL3 for tissue cultures of infected cells
Bacillus anthracis	Blood, cerebrospinal fluid, pleural fluid	Naturally and experimentally infected animals	Exposure of intact and broken skin, inoculation	BSL2, BSL3 in case of aerosol production, large quantities or high concentrations
Chlamydia psittaci	Blood, tissues	Birds, human	Exposure to infectious aerosols and droplets	BSL2, BSL3 in case of aerosol production, large quantities or high concentrations
Leptospira interrogans	Blood, tissues	Infected animals, human	Inoculation, skin and mucous membrane contact	BSL2
Listeria monocytogenes	Blood, cerebrospinal fluid	Naturally or experimentally infected animals	Ingestion, eye and skin exposure	BSL2
Mycobacterium atypicum	Bronchoalveolar lavage, lesion tissues	Human	Inoculation, direct skin contact, aerosol inhalation	BSL2
Mycobacterium tuberculosis	Gastric lavage, cerebrospinal fluid, pleural fluid, urine	Human, naturally infected primates	Aerosol inhalation	BSL3
Neisseria gonorrhoeae	Sinovial fluid, urine, cerebrospinal fluid	Human	Inoculation, direct skin contact	BSL2
Neisseria meningitidis	Pharyngeal exudates, bronchoalveolar lavage, cerebrospinal fluid, blood	Human	Inoculation, direct skin contact, aerosol inhalation	BSL2, BSL3 in case of aerosol production or high concentrations
Salmonella Salmonella typhi	Blood	Human	Inoculation, direct skin contact	BSL2, BSL3 for large quantities
Treponema pallidum	Lesion fluid	Humans with primary and secondary syphilis	Inoculation, direct skin contact, aerosol inhalation	BSL2
Toxoplasma, Trypanosoma, Leishmania, Plasmodium	Blood	Humans or experimentally infected animals	Inoculation, aerosol inhalation	BSL2
Blastomyces, Coccidioides, Histoplasma	Tissues	Infected animals	Inoculation, aerosol inhalation	BSL2, BSL3 for cultures containing Coccidioides, Histoplasma

This table was adapted from US HHS Publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999.

only contain pathogens, but may also be labelled with toxic and/or carcinogenic dyes, which create additional health risks for sorter operators (15–17).

Biological particles of 0.1–60 μm sizes (i.e., aerosols) were found to be important in the spread of infectious diseases (18–22). Submicrometer particles formed through dehydration of small droplets (droplet nuclei) can contain inorganic or organic material, or infectious agents and may stay suspended in air for prolonged periods. During inhalation, larger particles are deposited mainly into the nasal passages, particles in the size range from 3 to 7 μm into the tracheal area and pharynx, and $\leq$3 μm particles are deposited into the lung of the exposed individual (21). Droplets that fall out of suspension in air will fall onto surfaces, and any contained pathogens can then be transmitted to broken skin or mucous membranes, or by the naso-oral route. For instance, exposed eye mucosa creates a risk during laboratory work. Consequently, protection of all laboratory workers from exposure is critical, in particular during high-risk procedures such as droplet-based cell sorting on instruments using high-pressure systems.

Creation of droplets and aerosols during cell sorting. Jet-in-air technology used for cell sorting involves a liquid stream carrying the cells through a nozzle vibrating at a high frequency. At a given distance from the nozzle orifice, the stream is broken into individual droplets. These droplets are then passed between two high voltage plates. Droplets containing the cells that were preselected by the operator are electrostatically charged and deflected into sort sample receptacles. Overall droplet size depends on the instrument operating pressure and the size of the nozzle orifice and its vibration frequency. High-speed cell sorters utilize higher system pressures and sort frequencies (23), and thus they produce a higher number of smaller droplets compared with older instruments designed for low speed separations (24). All sorters also generate microdroplets, i.e., satellite droplets, 3–7 μm. Because of the high fluid pressure produced in high-speed cell sorters large amounts of secondary aerosols of various and undefined droplet sizes can occur during instrument failures, for example, when a partial clog in the nozzle causes a deflection in the fluid stream which splatters on a solid surface rather than into the trough of the waste catcher. Droplets larger than 80 μm constitute the majority of the droplets generated during sorting and they settle quickly out of the atmosphere, smaller droplets may be aerosolized, particularly when they are elevated by air currents. Because escaped aerosols are a potential health risk to sorter operators and the environment if aerosols escape into the room, aerosol containment of a free standing or enclosed cell sorter must be verified by using appropriate testing methods. Refer to the "Assessment of Aerosol Containment" section of this document for recommendations for the assessment of aerosol containment.

Laser hazards. The possibility exists that operators of cell sorters, during routine operation or optical alignment procedures, could become exposed to the intense, coherent, electromagnetic radiation emitted by high power lasers. Cell sorter operators must be sensitive to potential dangers to eyes and skin, as well as other fire, electrical, and chemical hazards. It is recommended that laser systems on cell sorters be operated according to the latest guidelines and national standards set in place by the governing bodies where the instrument is operated. In the United States of America (US), and for nations without adopted standards, it is recommend that lasers on cell sorters be operated according to the latest American National Standards Institute's (ANSI) Z136.1, American National Standard for the Safe Use of Lasers, and with other applicable regulations including the U.S. Federal regulations 21 CFR 1040.1 and 1040.11, U.S. Federal Laser Products Performance Standard and 29 CFR 1910, and the U.S. Occupational Safety and Health Standards for General Industry.

International Society for Analytical Cytology Biosafety Committee

In 1997 ISAC under the presidency of Joe Gray established a permanent Biosafety Committee with the goal of providing guidance to its membership and the scientific community at large in safety issues related to cytometry experimentation. Committee chair and current members are listed on the ISAC web site at: http://www.isac-net. org.

LEVEL OF CONTAINMENT

"Containment" refers to safe methods for managing infectious agents in the laboratory based on the type of organism and the type of risk because of the nature of the procedure. The four biosafety levels (BSL-1, BSL-2, BSL-3, and BSL-4) consist of a combination of laboratory facilities, laboratory practices and techniques, and safety equipment as outlined in the Center for Disease Control and Prevention Publication "Biosafety in Microbiological and Biomedical Laboratories," 4th Edition, 1999 (25), which is available on line at www.cdc.gov/od/ohs. Check periodically at this web address for updated versions of this publication. The purpose of containment is to reduce or eliminate exposure of biologically hazardous agents contained in processed samples to laboratory personnel, visitors, and the environment. Risk assessment of the experiments, performed on the available equipment, will define the appropriate combination of the different safety elements.

Considering the potential for exposure to aerosols during cell sorting, it is incumbent on the investigator who wishes to have such live, unfixed cells sorted or analyzed to determine the appropriate biosafety level (BSL). In conjunction with the flow cytometry laboratory director or manager and the investigator the sorter operator should review all the appropriate safety procedures for the particular pathogen for risk assessment. Such a review must also take into account all applicable regulations pertinent to gene technology. In the US these are described in the National Institutes of Health (NIH) Guidelines for Research involving Recombinant DNA Molecules (26). An extensive list of biological agents and their recommended BSL assignment is found in the CDC Publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999 and in other national or international regulatory documents (see below).

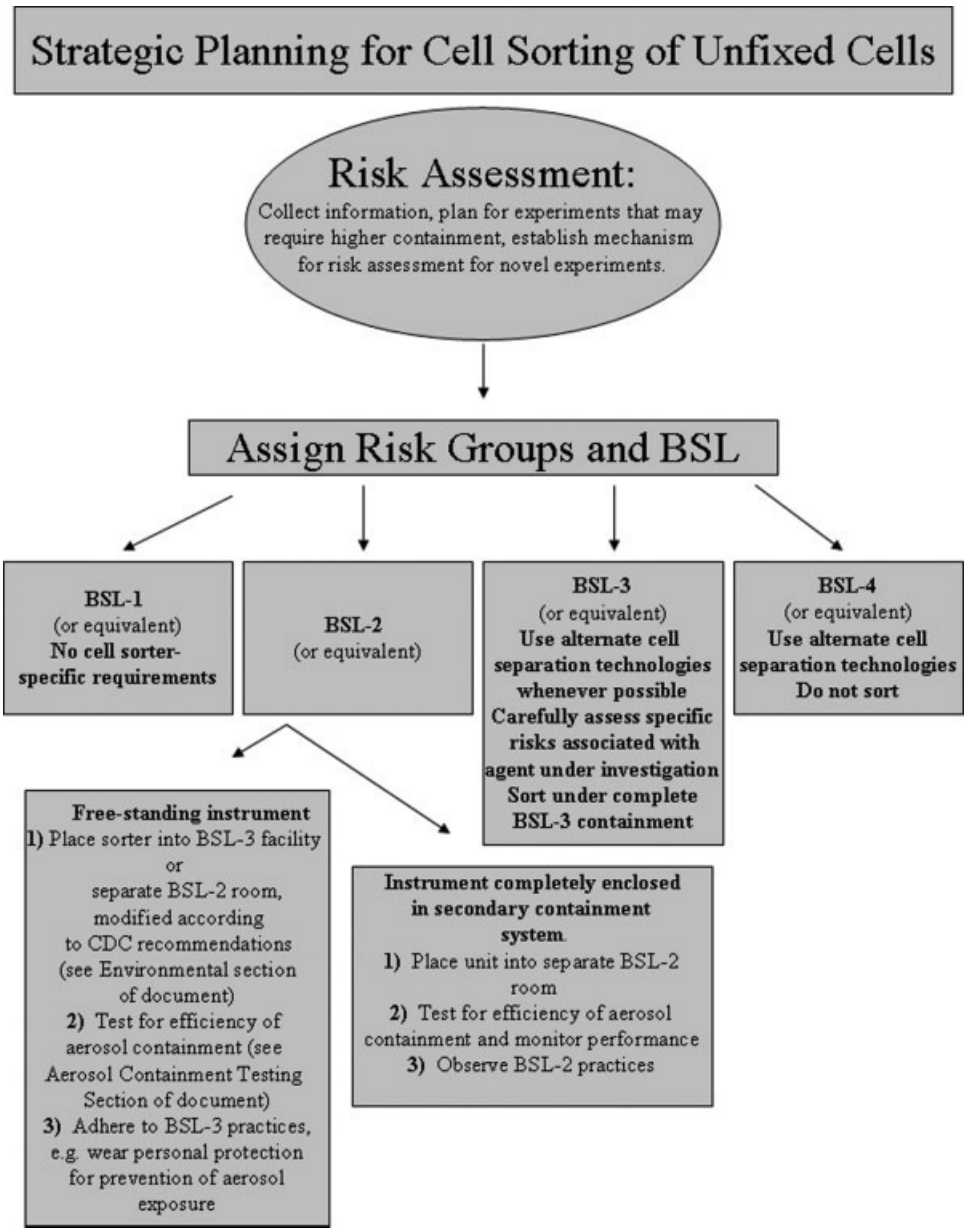
Strategic Planning for Cell Sorting of Unfixed Cells
Risk Assessment:
Collect information, plan for experiments that may require higher containment, establish mechanism for risk assessment for novel experiments.
Assign Risk Groups and BSL
BSL-1
(or equivalent)
No cell sorter-specific requirements
BSL-2
(or equivalent)
BSL-3
(or equivalent)
Use alternate cell separation technologies whenever possible
Carefully assess specific risks associated with agent under investigation
Sort under complete BSL-3 containment
BSL-4
(or equivalent)
Use alternate cell separation technologies
Do not sort
Free-standing instrument
1) Place sorter into BSL-3 facility or separate BSL-2 room, modified according to CDC recommendations (see Environmental section of document)
2) Test for efficiency of aerosol containment (see Aerosol Containment Testing Section of document)
3) Adhere to BSL-3 practices, e.g. wear personal protection for prevention of aerosol exposure
Instrument completely enclosed in secondary containment system.
1) Place unit into separate BSL-2 room
2) Test for efficiency of aerosol containment and monitor performance
3) Observe BSL-2 practices

FIG. 1. Diagram of the steps involved in achieving the appropriate containment level for performing cell sorting experiments using unfixed cells based on biosafety principles outlined in the CDC publication "Biosafety in Microbiological and Biomedical Laboratories," 4th edition, 1999, and other resources as described in the text.

Risk assessment is based on relative pathogenicity of the infectious or suspected infectious agent for healthy human adults. The investigator must make an initial assessment based on the risk group (RG) of an agent. Agents are classified into one of four risk groups. Risk group classification can be found in the "NIH Guidelines," Appendix B, on the American Biological Safety Association website under Resources, or may need to be determined. To determine the appropriate biosafety containment level the initial risk assessment should be followed by a thorough consideration of the agent itself and how it will be manipulated. For instance, manipulation of agents or biological materials using droplet-based sorting that leads to aerosol creation makes it necessary to use practices associated with a higher BSL to prevent exposure of personnel or the environment. Factors considered in determining the level of containment include virulence, pathogencity, infectious dose, environmental stability, route of spread, communicability, operations, quantity, availability of vaccine or treatment, and gene product effects. The containment level may be raised or lowered from the initial risk group classification only after thorough consideration. Refer to Figure 1 for an overview of strategic planning for sorting of unfixed samples.

Useful resources for conducting risk assessment are the Material Safety Data Sheets' at the Health Canada web site, http://www.phac-aspc.gc.ca/msds-ftss/index.html, American Public Health Association's "Control of Communicable Diseases," recent scientific articles and textbooks describing pathogenic agents or recombinant vector systems, and experts in the field (in the case of novel agents).

Clinical samples may contain unknown pathogens; in these cases, with the absence of hard data, a cautious approach and adaptation of a higher biosafety level is advisable. Risk assessment is likely to be most difficult for samples containing recombinant DNA molecules (27). In recent years technologies have evolved that lead to the generation of modified viruses, bacteria, yeast, and other microorganisms. Common recombinant viruses include adenoviruses, alphaviruses, retroviruses, vaccinia, and herpesviruses designed to express heterologous gene products. The challenge faced when selecting the appropriate biosafety level for such work begins by establishing the classification of the nonmodified virus and then proceeds to an evaluation for a possible increase in hazard potential associated with a given genetic alteration. Of particular concern are modifications that result in expression of a toxin or a known oncogene, or of sequences that alter the host range or cell tropism, or allow the virus to integrate into the host genome. If required, advice from a virologist should be sought to determine the proper BSL for planned flow cytometric experiments.

BSL-1 containment is used for work with agents not known to cause disease in humans. BSL-2 practices, safety equipment and facilities are used for pathogens that are known to cause disease in humans but can be easily contained and are not usually transmitted by aerosols. BSL-3 containment is applicable to biological agents that cause serious and potentially lethal disease as a result of exposure by the inhalation route. The ultimate level of containment, BSL-4, is reserved for work with exotic and highly dangerous organisms transmitted by aerosols for which no vaccination or therapies exist. These agents pose a high risk of life-threatening disease, therefore, viable sorts should never be performed when working with these pathogens. Refer to Table 1 for the recommended biosafety containment levels for selected agents and to Table 2 for the corresponding work practices.

At BSL-2 containment all laboratory manipulations that can generate infectious aerosols or aerosols that may pose a potential hazard must be performed in a Class I or Class II Biosafety Cabinet, while at BSL-3 containment all manipulations involving infectious or potentially hazardous materials must be conducted inside a biosafety cabinet. Many cell sorters do not fit within a biosafety cabinet; therefore, specimens are handled on the open bench during cell sorting. Furthermore, for any number of reasons, aerosol containment of a sorter may be breached during the cell sorting procedure and expose the operator to potentially biohazardous aerosols.

It is critical to understand when sorting any infectious or hazardous material, even if it is classified as BSL-2, that droplet-based sorting procedures are considered BSL-3 practices. Therefore, viable, unfixed samples that are potentially infectious must be sorted at a minimum on an instrument, which has been tested for aerosol containment (described in "Assessment of Aerosol Containment" section of this document), placed into an appropriately modified BSL-2 facility (for details refer to the Environmental Control section of the document) using practices and containment equipment recommended for BSL-3 practices according to the established guidelines outlined by the CDC (25). However, because of the increased hazard for a quick release of large amounts of fluid or aerosols into the environment, it is highly recommended that high-speed sorting be performed in a BSL-3 laboratory facility under complete BSL-3 containment.

Alternatively, containment systems that prevent aerosol escape by completely enclosing cell sorters into a Biocontainment Biological Safety Enclosure or into a biosafety cabinet will permit to sort BSL-2 classified materials using BSL-2 practices. However, BSL-2 practices are only allowed after rigorous initial testing in situ at installation and periodic retesting of these systems has validated their efficiency in containing aerosols during regular sorting, and during instrument failure modes using testing methods as described in the "Assessment of Aerosol Containment" section of this document. Monitoring devices that indicate proper operation of the biological safety cabinet during cell sorting are necessary.

RECOMMENDATIONS FOR CONTAINMENT CONTROLS

Standard Precautions and Other Regulatory Requirements

In the United States of America, all laboratory personnel who handle human cells and other potentially infectious materials such as specimens from experimentally infected animals are required to follow universal precautions. These procedures are outlined in the Occupational Safety and Health Administration document Occupational Exposure to Bloodborne Pathogens (28) and must be adhered to in addition to specific local and institutional safety regulations. Laboratories also must comply with federal code regulations for possession, use, and transfer of selected agents and toxins (29). All recombinant DNA experiments have to be performed in compliance with the specific NIH guidelines (26) and have to be approved by Institutional Biosafety Committees. All institutions receiving grant or contract awards from NIH are expected to follow the current health and safety guidelines published at http://grants1.nih.gov/grants/policy.

Other countries have developed their own stringent regulatory standards and/or have adopted aspects of regulations for work with biological agents as mandated in the US. International biosafety regulations, guidelines, and information sources are available online through the European Biosafety Association at http://www.ebsa.be/working_groups_international/biosafety_compendium.htm. Ex-

Table 2
Summary of Laboratory Practices Associated with Biosafety Levels

Biosafety levels	BSL-1	BSL-2	BSL-3
A. Hazard levels	Low risk	Low to moderate	Moderate to high
B. Standard microbiological practices			
Public access while experiments are in progress	Limited	Controlled	Not permitted
Handwashing facilities	Required	Required	Required
Eating, drinking, smoking, application of cosmetics, storing food for human consumption	Not permitted at any time	Not permitted at any time	Not permitted at any time
Pipetting	Mechanical devices	Mechanical devices	Mechanical devices
Safe handling of sharps	Required	Required	Required
Minimization of aerosol production	Recommended	Recommended	Required
Decontamination of work surfaces	Daily and upon spills	Daily and upon spills	Daily, upon finished work with infectious material, and spills
Infectious waste decontamination	Before disposal, placed in durable leak-proof container for transport	Before disposal, placed in durable leak-proof container for transport	Before disposal, placed in durable leak-proof container for transport
Biohazard sign	Posted at entrance	Posted at entrance	Posted at entrance
Insect/rodent control program	Required	Required	Required
C. Special practices			
Immunization	Not required	Recommended	Recommended; depending on the specific biohazard vaccination may be required
Medical surveillance (e.g., baseline serology, periodic testing)	Not required	Required when appropriate	Required when appropriate
Specific biosafety manual and training in policies and procedures appropriate for hazard potential	Not required	Required with annual up-dates	Required with annual up-dates
Bench top work	Permitted	Permitted	Permitted in some circumstances, but appropriate combinations of personal protective equipment, e.g., face-shields, respirators must be used
D. Safety equipment			
Laboratory coats, gowns, uniforms	Recommended, not worn outside the laboratory	Required, not worn outside the laboratory	Solid-front, wrap-around disposable clothing required for all workers with potential exposure to infectious agents
Gloves	Recommended, worn when skin contact with infectious material is unavoidable	Required when skin contact with infectious material is unavoidable; double gloving may be appropriate	Required when skin contact with infectious material is unavoidable; double gloving may be appropriate
Biological safety cabinets	Not required	Required for all aerosol generating processes	Required for all work with infectious agents
Other physical containment	Recommended that equipment be decontaminated immediately after use	Appropriate physical containment devices are used when procedures with high potential for creating infectious aerosols are being conducted[a]	Appropriate physical containment devices such as centrifuge safety cups , sealed centrifuge rotors are used for all activities with infectious materials that pose a threat of aerosols exposure[b]
Freezers/refrigerators	Recommended that biohazard sign be posted	Biohazard sign must be posted	Biohazard sign must be posted; all agents must be stored in separate, closed, labeled containers
E. Laboratory facilities			
Ventilation	No special requirements	Negative pressure	Ducted exhaust air ventilation system
Laboratory separated from the general public	Not required	Yes, while experiments are in progress	Required
Lockable doors	Not required	Required	Passageway with two self-closing doors
Sink	Recommended	Required	Required

Table 2
Summary of Laboratory Practices Associated with Biosafety Levels (continued)

Biosafety levels	BSL-1	BSL-2	BSL-3
Laboratory surfaces easy to clean, impervious to water, resistant to chemicals used for disinfection	Work surfaces	Work surfaces	Work surfaces, floors, walls and ceilings
Autoclave inside facility	Not required	Not required	Required
HEPA-filtered vacuum lines	Not required	Recommended	Required

This table was adapted from US HHS publication "Biosafety in Microbiological and Biomedical Laboratories," 1999.
[a]These procedures include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, opening containers of infectious materials whose internal pressures may be different from ambient pressures, and droplet-based sorting.
[b]These procedures include manipulation of cultures and of clinical or environmental material that may be a source of infectious aerosols.

amples include, but are not limited to the European Parliament-published Directive 2000/54/EC on the "Protection of workers from risks related to exposure to biological agents at work," which provides classification of biological agents into four risk groups and their associated containment measures (30), the Canadian Biosafety Guidelines, 3rd edition, the Handbook on the Regulation of Gene Technology in Australia, and the World Health Organisation Laboratory Biosafety Manual (31) available on line at http://www.who.int. Furthermore, guidelines for specimen handling based on US regulations that are focused on clinical settings are published in the document M29-A3 by the Clinical and Laboratory Standards Institute (32). Relevant details for the preparation of infectious samples containing HIV for flow cytometry, such as shipping and receiving of specimens, local transport, staining, and disposal, were previously described (33). Each laboratory must develop or adapt a biosafety operations manual, which specifies practices designed to minimize risks and takes into account the biohazard potential of the specimens that are processed (34). Appropriate safety practices are the responsibility of the laboratory director. Personnel must be trained in the required procedures and strict adherence to the techniques set forth is essential.

Handling of all unfixed human specimens and primary cell cultures as infectious is recommended (Universal Precautions). This practice also applies to established cell lines that are in vitro or animal-passaged human explanted tissues transformed by spontaneous mutation or a natural or laboratory infection with an immortalization agent, e.g., Epstein–Barr virus. In fact, cell lines from the American Type Culture Collection (ATCC) and other sources bear warnings that they may contain bloodborne pathogens, and ATCC recommends they be accorded the same biosafety level as the ones known to be infected with HIV. Likewise, specimens from nonhuman primates and animal tissues, explants, or cell cultures known to be deliberately infected with human pathogens are subjected to safety procedures as outlined in the Bloodborne Pathogen Standard.

Only rigorously characterized human cell lines that have been proved by stringent techniques, such as PCR, sensitive antigen detection, stimulation and coculture assays, enzyme analysis, etc., to be completely devoid of bloodborne pathogens could be excluded. In cases of genetically manipulated cell lines, researchers must demonstrate that the sample to be sorted is free of excess novel vectors or replication competent viral vectors prior to sorting the cells. However, as most laboratories are not able to provide reliable confirmation that the samples are pathogen–free, they are subjected to cell sorting using biosafety precautions as outlined in this standard.

If the samples are fixed, appropriate methods must be selected to reliably inactivate potentially bio-hazardous agents. Concerns exist about the effectiveness of standard fixation methods to reduce the level of infectivity in samples containing high titers of known viruses or unknown infectious agents resistant to inactivation (35,36). Fixation procedures must be performed carefully, otherwise, samples that are considered inactivated, but in fact are not, can pose a serious health risk to laboratory personnel.

Cell Sorter Operator-Specific Precautions

The protection of operators from infection and biohazard exposure during sorting of unfixed cells is of paramount importance (1,12,37–45). The following recommendations also apply to others who may be present in the room during the sort, e.g., scientists involved in the experiment.

Immunization. Whenever available, vaccination against a potential infectious organism that may be present in samples should be considered by the sorter operator. Vaccination against Hepatitis B virus is highly recommended.

Personal protective equipment. Sorting of unfixed samples on cell sorters that are not enclosed or incorporated into a biological safety cabinet requires personal protective equipment (PPE) for the operator. The PPE must conform to BSL-3 recommendations as outlined in the CDC Publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999. The sorter operator should wear a disposable, wrap-around, solid front, long sleeved laboratory coat made of fluid-resistant material. Examination gloves are required at all times, and in

some cases double-gloving should be considered. When the outer glove(s) are contaminated by contact with potentially biohazardous material, they must be sprayed with appropriate disinfectant and discarded. Then, new outer gloves should be worn over the inner gloves to prevent cross contamination. Torn gloves must be discarded and replaced with new undamaged gloves. In addition, laboratory manipulations cause gloves to fatigue; therefore, they should be changed often. It is recommended that the operator wear a respiratory protective device appropriate for aerosol protection, e.g., N95 NIOSH-approved particulate respirators covering nose and mouth, and safety glasses with side shields. For added splash protection, a full-face shield may be placed over the respirator and glasses. For an added safety margin operators may wear a complete body suit with a helmet and its battery-powered respiratory system with electrostatic filter media as described (40). Protective clothing is not worn outside the laboratory.

Specimen handling. All specimen processing prior to cell sorting should be performed in biological safety cabinets. Capped tubes or microtiter plates with sealed covers should be used as sample containers. For local transport, place primary collection tubes or sample tubes into a secondary container that is able to contain the specimen in case of breakage of the primary container, e.g., a plastic carrier with a secure lid. For specimen centrifugation, use sealed vessels or safety carriers.

1. Avoid use of "sharps": Avoid the use of needles, glass pipets, glass transfer pipets, or glass containers or tubes whenever possible for handling or transferring any biological material, and use suitable replacements. For disposal of any "sharps" a leak proof, puncture-resistant container as specified by local biosafety regulations must be used.
2. No mouth pipetting: No mouth pipetting is allowed. Manual pipetting devices must be used and must be equipped with filters to prevent infectious liquid from contaminating the pipetting device.
3. Sample preparation steps to minimize potential aerosol formation: Samples that are to be sorted need to be prepared as single cell suspensions because aggregated cells can partially or completely clog sort nozzles and stop a sort. Any interruption of a potentially biohazardous sort increases the risk of operator exposure to pathogens contained in the sort sample because of an increased probability for splashes and escape of sort aerosols during the manipulations required to continue sorting. To reduce the formation of cell aggregates during sample preparation, samples should be centrifuged gently, e.g., at ~300 g for 5–10 min. Higher centrifugation speeds can damage cells and compact them so densely that they are difficult to break apart. Frozen cell samples that are thawed for sorting frequently contain dead cells that may release DNA into the media. DNA binds to the surface of live cells, and after centrifugation these samples form solid aggregates leading to nozzle clogging problems and excessive aerosol formation. In these situations, addition of 20 μg/ml of RNase-free DNAse for 10 min at 37°C will prevent aggregation (45). Selection of an optimal solution for sample resuspension to maintain cell viability is important. Highly concentrated cell suspensions have an increased tendency to clump, therefore, dilute them to the lowest possible density for the sort speed used. Sort samples are often chilled to preserve cellular structures and prevent capping of antibodies bound to cell surface receptors. However, the cold can aggravate clumping, thus, keeping sort samples at an intermediate temperature such as 15°C may be preferable over 4°C. Immediately prior to sorting, all samples should be filtered through filter meshes with the appropriate pore size for the nozzle tip.
4. Work area clean-up: Work areas must be cleaned routinely. Discard all contaminated materials, e.g., sample and collection tubes, pipets, pipet tips, gloves, laboratory coats, into appropriate biohazard containers. Follow the established procedures at your institution for storage and disposal of biomedical/hazardous waste. Generally, this involves either autoclaving or decontamination with a 1/10 volume dilution of 0.71 M sodium hypochlorite (undiluted household bleach) prior to waste disposal. Wipe off all work surfaces with an appropriate disinfectant solution, taking into account the potential biohazard. Refer to Table 3 for information on the application of chemical disinfectants. Summary information on the survival and disinfectant inactivation of HIV has been published (46–49), and is reviewed in Ref. 33.
5. Disinfection of spills: After any spill of biological material, the protection of personnel is the first priority. In general, for small spills on a nonpermeable surface, a disinfecting agent, e.g., a 1/10 volume dilution of 0.71 M sodium hypochlorite (undiluted household bleach) is applied to a paper towel, placed on the spill, and allowed to make contact for an appropriate time to inactivate any biological organisms. Rapid clean up of spills should be an established laboratory practice. Refer to Table 3 for a summary of practical applications of chemical disinfectants. For the handling of larger spills or spills on a nonsmooth or permeable surface, refer to the Clinical and Laboratory Standards Institute document (M29-A3) (32) or the biosafety office of your institution.

Accidental exposure. It is recommended that all laboratory personnel provide a serum sample for storage as a baseline for future assay in the event of accidental exposure before their initial employment. Additional serum samples may be collected periodically, depending on the agents under study in the laboratory. Each laboratory must develop or adapt a written protocol to be followed in case of a suspected exposure to a biohazardous agent. Current guidelines for post exposure management of healthcare workers should be used and a medical evaluation, surveillance, and treatment record must be maintained (50–52). Guidelines for postexposure chemo-prophylaxis against HIV infection are available from CDC and should always follow the latest recommendations available on line at www.cdc.gov/mmwr.

Table 3
Summary of Practical Applications of Chemical Disinfectants for Decontamination

Chemical name	Quaternary ammonium compounds	Phenolic compounds	Chlorine compounds	Hydrogen peroxide	Iodophor	Alcohols		Aldehydes	
						Ethyl	Isopropyl	Form- (Formalin)	Glutar-
Practical requirements									
Use dilution	0.1–2%	1–5%	1/10–1/100 dilution of 0.71 M sodium hypochlorite, ~50–500 ppm[a]	3–6%	0.0075–10%, ~25–1600 ppm[a]	70–85%	70–85%	0.2–8%	2%
Contact time to lipovirus (min)	10	10	10	10	10	10	10	10	10
Broad spectrum	Not effective	Not effective	30 min	30 min	30 min	Not effective	Not effective	30 min	30 min
Inactivation profile									
Vegetative bacteria	✓	✓	✓	✓	✓	✓	✓	✓	✓
Lipoviruses	✓	✓	✓	✓	✓	✓	✓	✓	✓
Non-lipid viruses		–[b]	✓	✓	✓	–[b]	–[b]	✓	✓
Bacterial spores			✓	✓	✓			✓	✓
Fungi	–[c]	–[c]	✓	✓	✓	✓	✓	✓	✓
Protozoal parasites			–[d]					✓	✓
Characteristics									
Stability[e]	✓	✓		✓	✓	✓	✓	✓	✓
Corrosive		✓	✓	✓	✓				
Flammable						✓	✓		
Residue		✓	✓		✓			✓	✓
Organic material inactivated[f]	✓		✓	✓	✓				
Potential application									
Surfaces[g]	✓[h]	✓	✓	✓	✓	✓	✓	✓	✓[h]
Instrument surfaces and parts			✓	✓		✓	✓		
Flow cytometer fluid lines			✓	✓		✓			

This table was adapted from "Biohazardous Operations," Lawrence Livermore National Laboratory (1995) and from http://www.ianr.unl.edu/animaldisease/g1410.htm
[a]Available halogen; iodophors may require prolonged contact time for inactivation of bacterial spores, *Tubercle bacilli*, and certain fungi.
[b]Variable results depending on the virus; activity drops sharply when alcohols are diluted below 50%.
[c]Weak activity only.
[d]50% household bleach recommended for inactivation of *Cryptosporidium parvum* oocysts.
[e]Shelf life greater than 1 week when protected from light and air.
[f]Prior to decontamination cleaning with lipophilic detergent/disinfectant necessary.
[g]Work surfaces, decontamination of fixed or portable equipment surfaces.
[h]Usually compatible with optics, but consider interference from residues and effects on associated materials such as mounting adhesives.

Environmental Controls

Cell sorting of unfixed samples, especially when using sorters with high operating pressures, which are not enclosed into biological safety cabinets or biocontainment enclosures, should be performed in a BSL-3 laboratory facility because containment of the sorter could be breached any time during a sort because of a variety of reasons and release potentially hazardous materials into the area surrounding the instrument. A BSL-3 type room is only accessible through a passageway with self-closing lockable doors and requires a ducted HEPA-filtered air ventilation system. Additional requirements are as follows: water resistant interior surfaces (walls, floor, ceiling), laboratory furniture that can be easily cleaned and decontaminated, sealed windows, and a sink that can be operated without hands (Table 2). For further details, refer to CDC Publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999. Sort facilities in existing institutions may not have all the environmental safety features recommended for BSL-3. Sorting of unfixed cells can still be achieved in a BSL-2 laboratory facility provided the institution can modify the sorter room with the following requirements

- The air venting system discharges air towards the outside away from occupied areas or it is HEPA filtered.
- The cell sorter is located in a separate, lockable room where no other laboratory activity is performed.
- Air flow in the room is balanced to create airflow into the room with no less than 10 changes of air per hour. It is recommended that a visual monitoring device located at the door measure negative airflow.
- Access to the sorting room is restricted to allow the operator to concentrate on the sort and to maintain regular air flow and negative air pressure in the room. A sign should be placed on the outside of the door to indicate that a potentially biohazardous sort is in progress. This sign should also contain all necessary information, including warning for Class IV lasers, for entering the room safely, if needed.
- All the other safety recommendations for BSL-3 practices and personal protective equipment (PPE) are followed rigorously.

Compact cell sorters that can be placed into a biocontainment safety enclosure or biosafety cabinet, or larger sorters that have been integrated into a biosafety cabinet (53) allows sorting of BSL-2 materials using BSL-2 practices. However, they must be rigorously tested and carefully monitored for aerosol containment. Training in the operation and proper use of the containment device during sorting is mandatory.

Ultraviolet (UV) room light may be used to sterilize the room after each sort at the end of the day after all personnel have left, although its effectiveness against different pathogens may vary and areas where UV rays are blocked will remain unexposed. In addition, UV rays can damage some plastics. Therefore, routine cleaning with disinfecting agents is highly recommended.

Instrument Considerations

Proper operation of the cell sorter. Follow all the manufacturer's recommendations for instrument operation and maintenance carefully. Perform high-speed sorting of unfixed cells only on instruments that were designed for increased sorting rates or have been properly modified by the manufacturer. Never increase the system pressure on a cell sorter designed for low pressure sorting, because the fluidic lines, valves, and fittings cannot reliably withstand the increased pressure.

1. Maintenance: Set up a rigorous sorter preventive maintenance schedule either as part of a service contract offered by the manufacturer of the instrument or performed by laboratory personnel. Routinely perform leak checks on the fluid lines of the cell sorter. To do this, gain access to the fluidic lines. Carefully check for wet areas, indicating leaks in the tubing. Inspect tubing for cracks and signs of stress, particularly around the fittings and valve junctions. Also, inspect sheath lines and waste lines, and replace any leaking tubing.
2. Sort mechanism: Prior to each sort, verify the proper operation of the sort mechanism and the stability of the sort streams and droplet break-off. If the streams and the droplet break-off do not remain stable during the sort setup, correct the problem before sorting a potentially biohazardous sample.
3. Decontamination: After each sort, the instrument should be decontaminated with a disinfecting agent, taking into account the biohazards under study. Sort collection tube holders are heavily exposed to sample droplets and must be carefully decontaminated before handling. As per regulations outlined in the Bloodborne Pathogen Standard (28) appropriate disinfectants for decontamination of equipment and exposed work surfaces include diluted bleach, Environmental Protection Agency (EPA)-registered tuberculocides, EPA-registered sterilants, and products registered to be effective against HIV or HBV as listed on line at http://www.epa.gov/oppad001/chemregindex.html. Common laboratory disinfectants (4,54) applicable for instrument decontamination and their properties are listed in Table 3. Before designing a cell sorter-specific decontamination protocol the operator or laboratory manager should consult the instrument manufacturer for compatible disinfectants. Alcohols are not classified as high-level disinfectants, because they cannot inactivate bacterial spores and penetrate protein-rich materials, and isopropanol is not able to kill hydrophilic viruses. All surfaces inside the sort chamber, the sample introduction port and holder, are wiped down with appropriate disinfectant. Disinfectant is also run through the instrument for the appropriate exposure time and then followed with distilled water to completely remove the disinfectant as some disinfectants are corrosive to instrument components (consult manufacturer), and residual disinfectant solution can affect the viability of sorted samples. Make sure that the water used for removal of the disinfectant is sterile and does not introduce new contaminants into the instrument.

Sample introduction system. Cell sorters pressurize the sample tube once it is secured on the sample introduction port. While newer generation instruments are equipped with completely enclosed sample introduction chambers for operator safety, some older sorters have an open port requiring careful operator handling. Each time a sample tube is placed on the instrument, the operator must check the tube seal and its secure fit onto the sample introduction port. Otherwise, once the sample tube is pressurized, it could blow off and splash sample onto the operator or others involved in the experiment. Make sure that the tube material provides sufficient strength to tolerate high instrument pressure. On some instruments, when the tube is removed, the sample line back-drips, creating a potential biohazard through splattering of sample droplets on hard surfaces. To avoid this hazard, allow the back-drip to go into a tube until the sample is flushed out of its introduction line to avoid splashing of sample droplets. Alternatively, a soft absorbent pad soaked in disinfectant can collect the backdrip without splattering. Installation of a plastic shield around the sample introduction port can block droplet spraying from the sample back-drip. The catch tray or trough should be decontaminated carefully after each sort.

Nozzle tip. Since a clogged nozzle is one of the major reasons for increased aerosol production on cell sorters, samples should be prepared properly to minimize the formation of cell clumps. If clumped cells are present in the sample, they need to be removed. Options include filtration through nylon mesh filters, e.g., different pore size meshes, tubes with cell strainer caps, or individual cell strainers. Filter samples immediately prior to sorting to give cells less time to reaggregate. For large cell numbers distribute cell aliquots into separate tubes and filter each sample individually before placing it onto the sorter. If feasible, install a commercially available in-line filters, or filters made in the laboratory by heating the end of a clipped-off pipet tip and fusing it with nylon mesh, on the uptake port to prevent cell clumps from reaching the sort nozzle.

Select the appropriate nozzle size for the cell size to be sorted. Smaller nozzle sizes provide optimal signal resolution and easy sort setup, however, to avoid clogs, it is recommended that the nozzle orifice be at least four times larger than the cell diameter (55), but ideally it should be six times larger or even bigger. Sort nozzles should be cleaned frequently by sonication between sorts to prevent build-up of cellular debris. Contact the manufacturer to find out proper nozzle cleaning procedures as some nozzles will not tolerate sonication. Inspection under a microscope can help to determine if the nozzle is clear or damaged. However, cells can still aggregate inside a clean sort nozzle at the intersection of the sample fluid and sheath fluid, even during sorting of properly prepared samples. Accumulated cells at this intersection eventually break free and may partially clog the nozzle tip. When this happens, stop sample flow, turn off the high voltage, and put the fluidic control into the off position. Modern sorters have safety devices that will stop the sorting process as soon as a clog develops and cover the collection vessels. In any case, the sort chamber door must remain closed until aerosol has been cleared. Visual verification of the actual time needed for aerosol clearance should be performed with bottled smoke. Once a clog is detected, remove the sample to be sorted first. Then, only after aerosols have cleared, open the sort collection chamber door and take out the collection vials. Cap all tubes. Clear the clog by following the manufacturer's instructions. Before the sort is continued, make sure a straight and steady stream is emitted through the tip, and the droplet breakoff and side streams are stable.

Aerosol control measures. A standard safety feature of cell sorters includes an interlocking sort chamber door and a sample collection chamber designed to contain aerosols. These barriers are not always completely sealed and verification of air leaks should be performed with bottled smoke. Sealing of any such openings can aid in aerosol containment. All modern sorters are equipped with a receptacle that is connected with a waste evacuation system for collection of the undeflected center stream to reduce aerosol production. Auxilliary vacuum pumps designed to remove aerosols from the sort chamber have become available as optional attachments for sorters and are highly recommended. For custom installation of a generic vacuum pump care needs to be taken that no air turbulences are created that affect the stability of the sort streams. The vacuum lines for removal of airborne particles must be connected to a cartridge-type HEPA filter and a filter flask containing an appropriate disinfectant.

An efficient containment system on the sorter will be able to remove aerosols as long as the sort chamber door remains closed. If aerosol droplets escape, the efficiency of aerosol containment on the cell sorter must be verified in both regular sorting mode and instrument failure modes before sorting potentially biohazardous samples. If aerosol containment is incomplete, the safety features of the cell sorter must be modified so that no escape of aerosol can be detected. For compact sorters that are placed inside a biosafety containment cabinet or larger ones that are adapted to accommodate a biosafety cabinet as described (53), the efficiency of aerosol containment of the biological safety cabinet must also be verified.

Stream view cameras. Viewing cameras focused on sort streams are standard on newer sorters. They create distance between the sorter operator and the area of the sorter that poses the greatest potential biohazard. Viewing systems that illuminate the center stream and the deflected streams near the sort collection are recommended as they allow the operator to monitor increased aerosol production because of shifting stream positions and fanning.

LIMITATIONS AND ALTERNATE TECHNOLOGIES

Complete BSL-3 containment using the highest level of personal protection is required when agents to be sorted are known to be transmitted by the inhalation route. The greatest potential of exposure or infection from these agents is from aerosolization (e.g., *M. Tuberculosis*), and they are known to cause substantial morbidity and mortal-

ity. The need to perform such viable sorting experiments that should be reviewed on a case-by-case basis with the bio-safety office and infectious disease professionals of the institution. Cell fixation and alternate cell separation technologies, e.g., manual or automated magnetic bead separation or cell adherence to coated flasks that can be readily performed in biological safety cabinets should be used whenever possible. Future novel high-speed cell sorting technologies that do not generate aerosols, e.g., ultra rapid fluid switching technology, could offer a safer alternative to droplet-based sorting (24).

Samples labeled with radionuclides are posing major problems, as most sorting laboratories do not have authorization for using sources of ionizing radiation. In addition, the stringent requirements for handling such materials, including monitoring their use, disposal, instrument contamination, and generation of radioactive aerosols make flow cytometry experiments with radionuclide tracers not generally feasible.

TRAINING AND EXPERIENCE

Minimum Experience of Sorting Cells

Only experienced flow cytometry operators should perform potentially biohazardous sorts. The time required to obtain cell sorting proficiency on a given sorter varies, but training periods of 6 months are common. Some novel sorters do not require the complex alignment procedures required for older type instruments and laboratories do not feel a need for a dedicated instrument operator. *It is however extremely important that any operator who performs potentially biohazard sorting be trained carefully in the proper instrument operation and all relevant safety procedures, including aerosol containment testing on free-standing or compact sorters enclosed in biological safety cabinets. Strict adherence is mandatory as an operator error could invalidate aerosol control measures, endanger personnel and the environment.* The operator should have previous laboratory experience and a minimum of 2 years experience in flow cytometry. Ideally, this should include initial training on deflected-droplet cell sorters using noninfectious, fixed material of the same type that will contain the known biohazard, e.g., human peripheral blood mononuclear cell preparations, before sorting hazardous materials.

Experience with Potential Pathogens

Training and previous working experience with potentially biohazardous specimens are strongly recommended. Knowledge of the characteristics of common bloodborne pathogens and of the specific infectious organism present in the specimens to be sorted will aid the sorter operator to formulate safe working practices (56).

Mechanism for Institutional Evaluation and Approval

Each institution must establish a biosafety committee for the review of potentially hazardous laboratory protocols. A thorough review of the protocols and procedures for sorting of unfixed human cells will include but not be limited to the procedures used to establish containment of the cell sorter and an evaluation of the containment. Ad hoc review committees could be established for each application. These may consist of the investigator requesting the sort, the sorter operator, a representative of the biosafety office of the institution, and a scientist not involved with the protocol under consideration.

ASSESSMENT OF AEROSOL CONTAINMENT

The classic method for assessment of aerosol containment on deflected-droplet cell sorters using aerosolized bacteriophage and a detection system of bacterial lawns has been described in several publications (1,12,37,39). The T4 bacteriophage method for assessment of containment can also be combined with active air sampling for testing room air (1,38,39,45). The T4 settle plate method and the active air sampling method are described in detail in Appendix 1. Tagging aerosol droplets with bacteriophages is an established technique that, provided the titer of the bacteriophage is sufficiently high, insures that all droplets generated during the test sort contain T4. Because Merrill has established that a single phage is sufficient to generate one plaque (12) the assay provides high sensitivity. Furthermore, by counting plaques, the readout of containment results is straightforward. This method is an overnight procedure requiring intermediate knowledge of microbiological techniques and depends on the performance of biological materials.

Recently, a novel assay for measuring the efficiency of aerosol containment has been described (42,43). The method uses a suspension of highly-fluorescent melamine copolymer resin particles, which simulate a biological sample during the test sort. Aerosol containment is measured by placing microscope slides around the instrument where aerosols are produced and could escape. The slides are examined for the presence of particles under a fluorescent microscope. Perfetto et al. (40,44) have increased the assay's sensitivity and reproducibility by using a viable microbial particle sampler. This device draws room air onto a microscope slide and concentrates the collected resin particles onto the areas on the slide located directly underneath the intake ports. This technique is described in Appendix 1 and is suitable to be performed immediately before a potentially biohazardous sort; however, for this practice a fluorescent microscope has to be readily accessible. The highly fluorescent particles can be easily detected, but careful handling of the microscope slides, and the air sampler are important to avoid false positives. Diligent scanning of the entire slide is required to reliably detect escape of a single particle.

Before sorting any unfixed and potentially bio-hazardous specimens on a given instrument, it is imperative to validate that aerosols are contained during the regular sorting process and during instrument failure modes [Appendix 1]. If aerosols are detected outside of containment, then the cell sorter must be modified such that no aerosols are detectable. Contacting the instrument manufacturer for instructions or dispatching a service engineer

will be necessary before making any instrument modifications. Testing must also be done whenever changes are made to the cell sorter that may affect escape of aerosols, e.g., installation of a new drive head or flow cell, replacement of the sort chamber door, or alterations in the aerosol management system.

For instruments that are placed into biological safety cabinets, it is imperative that laboratories validate initially at installation the efficiency of aerosol containment of the cabinet before any potentially bio-hazardous sorting experiments are performed. Frequent retesting and monitoring proper functioning of the cabinet is mandatory.

Since every live infectious sort has the potential to create infectious aerosols, verification of aerosol containment should be performed as often as possible. It is strongly recommended to perform testing prior to every infectious cell sort and maintain a record of the results. This practice will assure validation of the aerosol management system to contain aerosols containing potentially infectious pathogens.

CONCLUSIONS AND NOVEL APPLICATIONS

Laboratories involved in basic or clinical research are faced with increasing demands for sorting of unfixed samples. Biohazardous sorting is often performed for infectious disease studies to separate leukocyte subsets based on cell surface expression patterns. Sorted cell populations can then be examined for their response to the pathogen of interest, for cellular mechanisms of its pathogenesis, or for identification or characterization of cells infected with the pathogenetic organism (37). Recent sorting applications include studies of gene expression in cells that either carry a pathogen or have been transfected with fluorescent vectors that contain genetic sequences of an infectious agent (57).

Novel applications involve preparative cell sorting of clinical samples for therapeutic interventions (58–61). Clinical cell sorting requires protection of instrument operators from known or unknown pathogens contained in the patient samples using practices as outlined in this standard. In addition, preparative sorting of any product infused into patients has to be performed using Good Manufacturing Practices (GMP). GMPs help assure product quality largely by preventing contamination through specific activities such as environmental conditions (e.g., clean room), decontamination practices and testing (23,62,63).

The prevention of exposure of laboratory personnel to biohazards is of great importance. Although up to this point in time no infection because of sorting of unfixed material has been documented, the updated recommendations set forth here represent a timely effort of ISAC to provide a set of standards for sorting of unfixed cells, including known biohazardous samples. These recommendations may also aid laboratories in obtaining institutional (e.g., Institutional Biosafety Committee, Institutional Review Board) and/or regulatory agency approval for sorting of such unfixed cells. Furthermore, it is hoped that these standards will continue to prompt cell sorter manufacturers to design new instruments with operator and environmental safety in mind.

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APPENDIX: TESTING OF AEROSOL CONTAINMENT ON CELL SORTERS

Introduction

This section contains laboratory protocols developed for aerosol containment testing of sorters. The T4 bacteriophage protocol written by Ingrid Schmid was developed in the laboratory of the late Dr. Janis V. Giorgi and incorporates work performed by Lance Hultin and Dr. John Ferbas. It was expanded from previous publications (12,37,38) and has been published in more detail in a chapter in Current Protocols in Cytometry (39) and in a recent chapter (45). The bead-based aerosol testing method described here was developed by Stephen Perfetto and others in the laboratory of Dr. Mario Roederer (40,44) and incorporates work described in previous publications (42,43).

Purpose

As part of the normal sorting process, deflected-droplet fluorescence-activated cell sorters produce droplets and microdroplets. Secondary aerosols are produced when the fluid streams splash into receptacles and when a clogged

nozzle causes a deflection in the streams that is striking an obstacle. Droplets that are smaller than 80 μm can be aerosolized and could escape into the area surrounding the instrument. If these aerosols harbor active infectious agents they pose a potential hazard to the operator. Therefore, the effectiveness of aerosol control measures on commercially available cell sorters has to be evaluated during routine operation and in failure mode. Furthermore, any jet-in-air flow cytometer even when it is not used for cell sorting can produce aerosols, particularly, when the nozzle is partially clogged; therefore, it is advisable to also test instruments that are used for data acquisition of unfixed samples. As most of the aerosol mass produced by cell sorters consists of droplets of >5 μm size and thus quickly settles from air through gravity, the settle plate method described below will effectively measure aerosol escape. However, cell sorters can also produce aerosolized droplets of ≤5 μm sizes (droplet nuclei). Droplet nuclei can stay suspended in air for prolonged periods of time and can be deposited into the lung of the exposed individual during inhalation. If testing of the instrument for escape of droplet nuclei is desired an active air sampling method has to be used. The T4 bacteriophage assay has high sensitivity because it has been established that one phage is sufficient to create a plaque and the readout is straightforward. However, intermediate knowledge of microbiological techniques is required, preparation of the biological materials for the test takes several days, and results require overnight incubations.

Several years ago a nonbiological method for measuring aerosol escape was developed (43) using highly fluorescent melamin copolymer resin particles (Glo Germ™, Moab, Utah). Perfetto et al. modified the original method (40,44). A suspension of these particles is run through the instrument at speeds and concentrations simulating a biological sample. Aerosol containment is tested by placing microscope slides into a vacuum-driven air device designed to efficiently sample aerosols from air. This device can be positioned around the instrument testing a variety of areas for potential aerosol escape. Slides are then examined under a fluorescent microscope for the highly fluorescent resin particles. Readouts produce a "real-time" quantitative result of containment and therefore are suitable to demonstrate protective containment before each infectious sort is performed. However, this method is also applicable to instrument validation and periodic testing.

Bacteriophage and *E. Coli* Settle Plates

Testing room air with bacteriophage and an active air sampling method. A broth culture of *Escherichia coli* (*E. coli*) is set up to create confluent bacterial lawns on petri dishes. These dishes are placed in the working environment while T4 bacteriophage are run through the instrument and are aerosolized. Plaques formed by the bacteriophage landing on the bacterial *E. coli* lawns and lysing the bacteria are used to indicate the presence of aerosol. For testing of room air, petri dishes with confluent lawns of *E. coli* are placed into a single-stage N6 Andersen air sampler. Air is drawn onto these dishes while T4 bacteriophages are run through the cell sorter. Bacteriophage plaques formed on the lawns indicate the presence of droplet nuclei (aerosolized droplets of <5 μm sizes) in the room air. The procedures are relatively simple but must be followed closely to insure the success of the experiment. Although concerns exist about utilization of *E. coli* in an environment where sterile cell sorting is to be performed, our laboratory has not encountered problems with the sterility of sorted samples since we have started to test our instruments for aerosol containment. No plaques should be detected on the petri dishes placed outside the sort chamber and on petri dishes that were collected from the air sampler. Otherwise, the instrument has to be modified to achieve aerosol containment. Repeat testing every 1–3 months.

Preparation of materials. *Media: All media ingredients are per liter.*

- Nutrient broth: (a) Bacto nutrient broth (Difco 0003-02-05), 8.0 g; (b) sodium chloride, 5.0 g; (c) glucose, 1.0 g; Adjust the pH to 7.2–7.4 with 1N NaOH before autoclaving for 15 min, 121°C.
- Dilution broth: (a) Bacto tryptone (Difco 0123-15-5), 10.0 g; (b) sodium chloride, 5.0 g; Adjust the pH to 7.2–7.4 with 1N NaOH before autoclaving for 15 min, 121°C.
- Top agar: (a) minimal agar Davis (Difco 0544-17-4), 6.5 g; (b) Bacto tryptone, 13.0 g; (c) sodium chloride, 8.0 g; (d) sodium citrate (dihydrate), 2.0 g; (e) glucose, 3.0 g; Adjust the pH to 7.2–7.4 with 1N NaOH before autoclaving for 15 min, 121°C.
- Bottom agar: (a) minimal agar (Davis), 10.0 g; (b) Bacto tryptone, 13.0 g; (c) sodium chloride, 8.0 g; (d) sodium citrate (dihydrate), 2.0 g; (e) glucose, 1.3 g; Adjust the pH to 7.2–7.4 with 1N NaOH before autoclaving for 15 min, 121°C.

T4 bacteriophage stock preparation.

- Rehydrate one vial of ATCC 11303 *E. coli* with 0.3–0.4 ml of nutrient broth and mix well. Transfer 0.1 ml of this suspension to a sterile 25 ml Erlenmeyer flask containing 10 ml of nutrient broth. Put a streak onto nutrient agar slants for short term storage (6 months). The rest of the *E. coli* suspension can be frozen at −20°C for long term storage.
- Incubate the flask on an orbital shaker (~150 rpm) at 37°C overnight. Also incubate the slants overnight at 37°C.
- Subculture 0.5 ml of the broth culture into sterile 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth. Incubate the flasks on an orbital shaker at 37°C until the culture enters log phase, or becomes slightly turbid. Refrigerate the slants for short term storage at 4°C (6 months).
- Rehydrate the lyophilized T4 bacteriophage (ATCC 11303-B4) with 0.5 ml of nutrient broth. Mix well and in-

oculate 0.1 ml of phage suspension into the log phase cultures of *E. coli* prepared as described earlier. Refrigerate the remaining bacteriophage suspension for short term storage at 4°C (6 months).

• Incubate the *E. coli*/T4 bacteriophage cultures at 37°C until lysis of *E. coli* is complete as indicated by clearing of the turbid suspension. This usually takes about 12 h, however, the cultures may be incubated overnight if they are started in the afternoon.

• Transfer the broth from the flasks with sterile 10 ml pipets into sterile 50 ml chloroform-resistant centrifuge tubes. Add 12 drops of chloroform to each tube and shake vigorously. Remove cellular debris by centrifugation at 2,000×*g* for 20 min.

• This bacteriophage stock solution can be refrigerated in a tube with minimal airspace indefinitely. The titer should be periodically determined to document that the titer has not decreased. The expected titer is $>1 \times 10^9$ plaque forming units (PFU)/ml and is determined by serial dilution as described below.

• The bacteriophage may be re-expanded, as needed, by inoculating a log phase *E. coli* culture by repeating this procedure, except that the bacteriophage is passed from the stock solution.

Preparation of bacterial lawns.

a. About 1 week prior to the aerosolization experiment: prepare agar plates.

• First, the bottom agar is liquified (this can be done conveniently in a microwave oven, use an adequate container to prevent boil-over), then, ~20 ml are pipetted into each of twenty-three 100-mm diameter petri dishes and allowed to solidify (23 plates are prepared because 6 are needed for titration of phage, 12 for the verification of aerosol containment on the flow cytometer, and 5 extra in case of an accident); six more are needed if a test with the air sampler is to be performed. The preparation of these plates (as well as the other procedures in this protocol) may be performed on the benchtop, provided that good aseptic technique is followed. After adding the liquified agar to the plates, leave the lid ajar until the plate cools.

• Once the agar has cooled and solidified, store the plates upside down. The plates may also be placed in a plastic bag to prevent dehydration and stored in a refrigerator or cold room for up to 6 months. The plates should be visually inspected for contamination so that new plates may be prepared prior to the experiment, if needed.

b. About 18–24 h prior to the aerosolization experiment: Set up a broth culture of *E. coli*.

• Using an inoculating loop (or sterile plastic pipet) transfer some *E. coli* from the agar slant to 100 ml of nutrient broth in a 250 ml sterile Erlenmeyer flask.

• Grow the *E. coli* at 37°C overnight on an orbital shaker. Use a sterile gauze pad to plug the Erlenmeyer flask.

c. The day of the experiment: combine *E. coli* with liquified "top agar" for the preparation of the plates that are used in the actual aerosol containment experiment on the flow cytometer.

• Heat ~110 ml top agar in a microwave and allow it to cool to between 40 and 50°C. Monitor its temperature by placing an ethanol-sterilized thermometer into the liquified agar.

• Add ~2 ml of the *E. coli* broth culture to the top agar, swirl gently, and add 4 ml of this culture to 15 petri dishes (12 plus 3 extra) prepared with bottom agar (see above). Six more dishes are needed if room air is tested with the air sampler.

• Once all the plates are completed and the top agar has solidified, move the plates to a 37°C incubator or warm room to allow the *E. coli* to grow. Keep the plates level at all times so that the top agar remains intact.

• Allow the *E. coli* to grow in these plates for 1–2 h before using them in the aerosolization experiment. The plates should have a slightly opaque appearance after the 1–2 h incubation because the *E. coli* will begin to form a confluent lawn.

Preparation for the aerosolization experiment—titration of the T4 bacteriophage. During this procedure, the bacteriophage stock is titrated, the throughput of bacteriophage per unit time on the flow cytometer is determined, and the viability of the bacteriophage stock after it is run through the instrument is assessed.

Titration of the bacteriophage stock.

• Follow the corresponding schematic for performing serial dilutions of T4 stock.

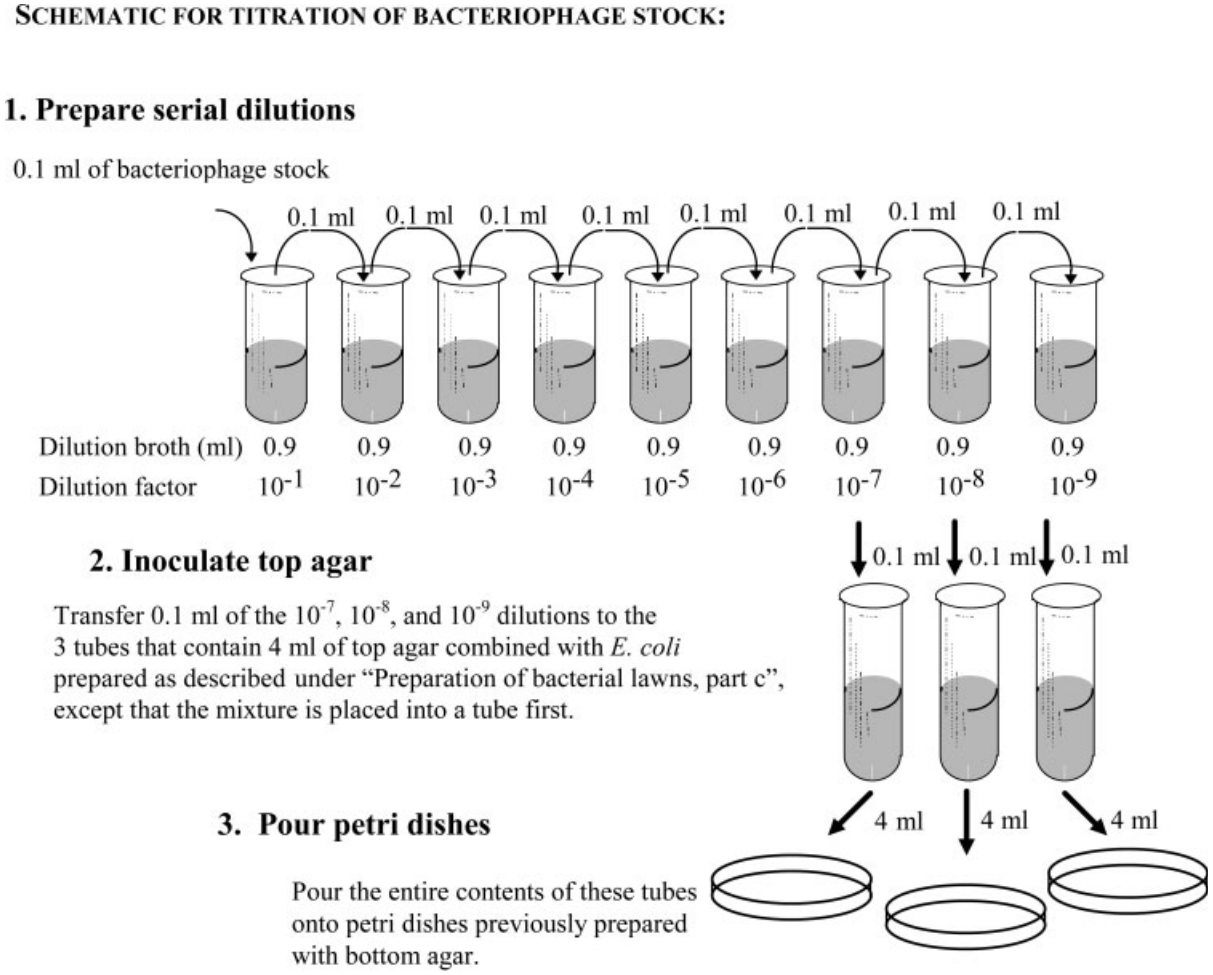
• Once the three plates are inoculated and the top agar has solidified, move the plates to a 37°C incubator or warm room to allow the *E. coli* to grow overnight. The following day, plaques will appear on the bacterial lawns. By counting the plaques, the actual plaque forming units per milliliter of bacteriophage stock can be determined. Note that the lower dilutions are discarded in the above schematic because they will show confluent lysis of the bacterial lawns. However, if the titer of the bacteriophage stock is lower, some or all of these dilutions may have to be put onto plates to achieve appropriate numbers of plaques.

Example of results:

Dilution	Number of plaques
10^{-7}	TNTC^
10^{-8}	40*
10^{-9}	1

^Too numerous to be counted.

*Select a petri dish with an intermediate number of plaques (usually between 10 and 100) and correct the value by the final dilution factor. Note that the final dilution factor in this example actually reflects a dilution factor of 10^{10} (not 10^8), because initially only 0.1 ml of the phage suspension was taken, then serially diluted, and finally 0.1 ml was added to the top agar, e.g., 40 plaques $\times 10^{10} = 4 \times 10^{11}$ PFU/ml.


SCHEMATIC FOR TITRATION OF BACTERIOPHAGE STOCK:
1. Prepare serial dilutions
0.1 ml of bacteriophage stock
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
Dilution broth (ml) 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9
Dilution factor 10-1 10-2 10-3 10-4 10-5 10-6 10-7 10-8 10-9
2. Inoculate top agar
Transfer 0.1 ml of the 10-7, 10-8, and 10-9 dilutions to the 3 tubes that contain 4 ml of top agar combined with E. coli prepared as described under "Preparation of bacterial lawns, part c", except that the mixture is placed into a tube first.
0.1 ml
0.1 ml
0.1 ml
3. Pour petri dishes
Pour the entire contents of these tubes onto petri dishes previously prepared with bottom agar.
4 ml
4 ml
4 ml

Determination of bacteriophage throughput on the flow cytometer. Bacteriophage throughput on the cell sorter is determined to insure that an adequate number of viable, infectious organisms are present within the sorting aerosol. For assessment of throughput, it is necessary to know the sample flow rate per unit time. The *expected* and *actual* throughput is measured to validate an inordinate number of bacteriophage are not destroyed during passage through the instrument.

a. Calculation of the *expected* throughput:

- Record the weight of exactly 1 ml of bacteriophage suspension in a tube that will be used for the sorting experiment, e.g., 1.0 g.
- Run the sample through the instrument under normal sorting conditions for 10 min, and reweigh. e.g., 0.6 g.
- Determine the sample throughput, where 1 mg = 1 μl, e.g., 1.0 g − 0.6 g = 0.4 g or 400 μl → 400 μl/10 min = 40 μl/min.
- Calculate the expected number of bacteriophage flowing through the instrument, e.g., where the concentration of bacteriophage is 4×10^{11} PFU/ml. 40 μl/min $\times$ 4×10^{11} PFU/1,000 μl = 1.6×10^{10} PFU/min.

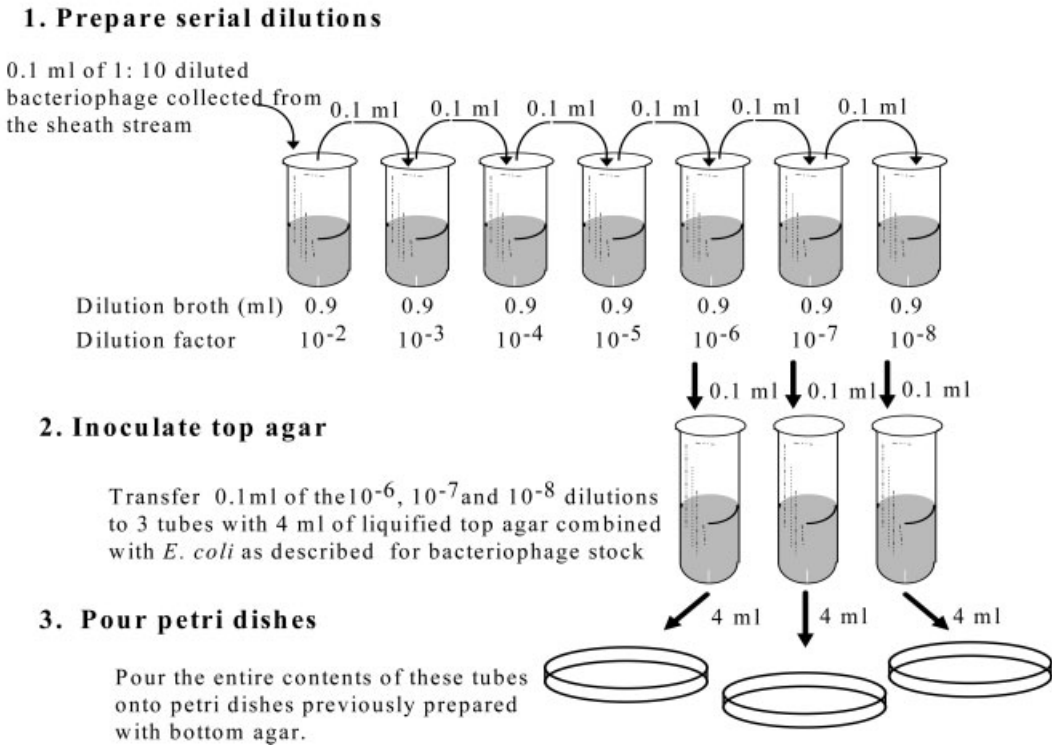
b. Measurement of the *actual* throughput:

Here, the number of bacteriophage flowing through the instrument is measured directly from the sheath stream as it exits the nozzle tip.

- Collect the sheath stream for exactly 1 min while running bacteriophage through the instrument into a tube that has been weighed empty. Reweigh it to determine the volume collected in 1 min, e.g., 2.35 ml.
- Immediately transfer 0.1 ml of the sample to 0.9 ml of dilution broth. This is important because the bacteriophage in the sample needs to be titered to determine throughput, and bacteriophage may lose infectivity in many sheath fluids as a function of time. This sample can be titered immediately as described below. Alternatively, it may be held in the dilution broth at ambient temperature until the aerosolization experiment is completed.

Titration of bacteriophage run through the flow cytometer. Follow the corresponding schematic for performing serial dilutions of T4 in sheath fluid. Once the three plates are inoculated and the top agar has solidified, move the plates to a 37°C incubator or warm room to allow the *E. coli* to grow overnight. The following day,

SCHEMATIC FOR TITRATION OF BACTERIOPHAGE IN SHEATH FLUID:


1. Prepare serial dilutions
0.1 ml of 1: 10 diluted bacteriophage collected from the sheath stream
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
Dilution broth (ml) 0.9 0.9 0.9 0.9 0.9 0.9 0.9
Dilution factor 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}
0.1 ml
0.1 ml
0.1 ml
2. Inoculate top agar
Transfer 0.1ml of the 10^{-6}, 10^{-7} and 10^{-8} dilutions to 3 tubes with 4 ml of liquified top agar combined with E. coli as described for bacteriophage stock
3. Pour petri dishes
4 ml
4 ml
4 ml
Pour the entire contents of these tubes onto petri dishes previously prepared with bottom agar.

plaques will appear on the bacterial lawns. By counting the plaques, the actual plaque forming units per unit time (actual throughput) can be determined. Note that the lower dilutions are discarded in the earlier schematic because they usually will show confluent lysis of the bacterial lawns. However, if the titer of the recovered bacteriophage suspension is lower, some or all of these dilutions may have to be put onto plates to get intermediate numbers of plaques.

Example of results:

Dilution	Number of plaques
10^{-6}	40*
10^{-7}	1
10^{-8}	0

*To calculate the actual throughput, select a petri dish with an intermediate number of plaques (usually between 10 and 100) and correct the value by the final dilution factor. Note that the final dilution factor in this example actually reflects a dilution factor of 10^7 (not 10^6) because 0.1 ml of the sample was added to the top agar. Then, the total number of phages collected in 1 min is calculated by multiplying the result with the volume in the tube, e.g., (40 plaques $\times$ 10^7/0.1 ml) $\times$ 2.35 ml = 9.4 $\times$ 10^9 PFU/min.

Testing the Flow Cytometer for Aerosol Containment Using Settle Plates. Testing the flow cytometer for aerosol containment is performed in two parts. First, bacteriophage is run through the instrument for 2 h using instrument settings that approximate the maximum sorting rate that could be used during cell sorting. This condition is referred to as the "good" mode. Once completed, the sorter is adjusted to produce maximal fanning of the sort streams to produce as much aerosol as possible and run for 15 min. This condition is called "bad" mode.

- For the good mode, six petri dishes with confluent *E coli* lawns are used. Two are placed within the sorting chamber, near the sorting streams. Two are placed immediately outside of the sorting chamber door, and the remaining two plates are placed somewhere near the instrument in the room. It may be necessary to jerry-rig a level platform for the dishes that are placed inside of the sorting chamber.
- After removing the lids from the plates, and closing the sorting chamber door, initiate the mock sort. Bacteriophages are run through the instrument and sorting streams are generated according to the instrument-specific software with ~1,000 sort decisions per sec each left and right. Approximately 3.5 ml of bacteriophage suspension is needed.
- Run the good mode for 2 h. Replace the collection vials when they are full. Wait for ~3 min for aerosol clearance before opening the door. Place lids onto the petri dishes that are placed outside the door to avoid false positive plaques on the dishes outside the door.
- When the good mode is completed, replace the lids on each plate and incubate the plates at 37°C overnight for plaque development.

Date:

T4 BACTERIOPHAGE SORT FOR TESTING OF AEROSOL CONTAINMENT

I. CALCULATION OF SAMPLE RATE (µl/min)

Volume fed to machine (µl): -_____ Feeding time (min): 10 min

Sample rate (µl/min): _____

Does flow rate meet established criteria (30-40µl/min)? YES ☐ NO ☐

II. TITRATION OF T4 BACTERIOPHAGE STOCK (MINIMUM 10^9 PFU/ML)

Plate ID	Dilution	# of plaques	Dilution factor	Vol on plate	PFU/ml
A1	10^{-7}				
A2	10^{-8}				
A3	10^{-9}				

Does the T4 phage stock meet minimal concentration requirement (10^9 PFU/ml)? YES ☐ NO ☐

III. CALCULATION OF EXPECTED T4 FLOWING/MIN THROUGH CELL SORTER

Sample rate: µl/min T4 stock conc: PFU/ml Expected T4 throughput: PFU/min

IV. T4 FLOWING/MIN TROUGH CELL SORTER (MINIMUM 10^7 PFU/MIN)

Collect sheath stream for 1 min

Volume collected (ml): _____ml

Plate ID	Dilution	# of plaques	Dilution factor	Vol on plate	Sheath rate	PFU/min
B1	10^{-6}					
B2	10^{-7}					
B3	10^{-8}					

Does the T4 phage stock meet optimal established criteria for flow rate through flow cytometer (>10^7 PFU/min)? YES ☐ NO ☐

V. AEROSOL CONTAINMENT IN GOOD SORTING MODE

Plate ID	Plate location	# of plaques	Time
C1	inside right		2 h
C2	inside left		"
C3	door right		"
C4	door left		"
C5	x adjust		"
C6	top of machine		"

INSTRUMENT SETUP

Sheath pressure	psi
Drop frequency	KHz
Sort rate	decision/s
Droplets sorted	
Sample flow rate	µl/min
Door closed	
Vacuum on	
Nozzle tip	µ m
Auxiliary vacuum	
Drop drive amplitude	volts

Is aerosol contained in good sorting mode? YES ☐ NO ☐

VI. AEROSOL CONTAINMENT IN BAD SORTING MODE

Plate ID	Plate location	# of plaques	Time
D1	inside right		15 min
D2	inside left		"
D3	door right		"
D4	door left		"
D5	x adjust		"
D6	top of machine		"

Is aerosol contained in bad sorting mode? YES ☐ NO ☐

REMARKS: _____Signed:

• Next, the bad mode is performed with six new petri dishes with confluent *E. coli* lawns. The bad mode is run in the same manner as the good mode, except that the sorter is adjusted to produce sort stream fanning for the duration of 15 min (the bad mode is created by turning off the droplet drive).

• Record test data. See an example form shown here.

Testing room air for escape of droplet nuclei using an active air sampling method. Testing the cell sorter for aerosol containment of droplets ≤ 5 μm (droplet nuclei) is performed in combination with the T4/*E. coli* settle plate test.

• Before T4 bacteriophage are run through the cell sorter, set up the N6 Andersen single-stage air sampler (Graseby–Andersen, GA, or equivalent), and place one petri dish with a confluent *E. coli* lawn with the lid off into the sampling stage. Collect room air for 10 min. This dish will serve as negative control.

• Place two *E. coli* plates each into a sampling stage. Place one stage close to the cell sorter, e.g., where the operator is sitting and the second one ~1 m away from the instrument. Collect room air during the last 10 min of the good mode containment test.

• Place two *E. coli* plates each into a sampling stage. Place one stage close to the cell sorter and the second one ~1 m away. Collect room air during the last 10 min of the bad mode containment test.

• Also, sample room air for 10 min during the bad mode containment test with the sort chamber door open. This plate will serve as positive control.

• After the completion of each test, replace the lids on all the petri dishes and incubate them overnight at 37°C for plaque development.

• Record all data.

Troubleshooting.

• *E. coli lawns are not apparent after overnight incubation.* When *E. coli* is added to top agar in excess of 50°C, the bacterium may be killed. Monitor the temperature of the top agar prior to adding the *E. coli.*

• *The liquified agar solidifies too fast to prepare all the petri dishes.* Maintain the liquid state indefinitely in a 50°C water bath.

• *Plaques are not observed in the sample collected from the sheath stream.* Bacteriophage may be incompatible with the sheath fluid that was used (e.g., PBS is an incompatible, HBSS is an acceptable sheath fluid). Note also that sheath fluids containing anti-bacterial agents, e.g., gentamycin, are not advised for these experiments. For verification, plate lower dilutions of the sheath stream on the *E. coli* lawns (as described in the above schematic). The measured titer should be $>10^7$ PFU/min. If the titer is much lower, suboptimal conditions may have been used.

• *Plates placed inside of the sorting chamber do not develop plaques, but rather have large and uneven areas of complete lysis.* The plates placed within the sorting chamber are used to measure aerosols from the sorting streams, and not the sorting streams themselves. If the plates are placed too near to the sorting streams, the plates will get wet and will be inoculated with sheath fluid rather than aerosols.

Testing Aerosol Containment using Glo Germ™ Particles and a Microbial Particle Sampler

Testing is performed before each biohazardous sort. The instrument is placed in failure mode to simulate conditions, such as a partially blocked sort nozzle or air in the fluid system, which result in a marked increase of aerosol production. Aerosol containment results are recorded as PASS/FAIL before each sort.

Materials and methods.

1. AeroTech 6TM viable microbial particle sampler, Cat. No. 6TM (AeroTech Laboratories, Phoenix, AZ) (www.aerotechlabs.com).
2. High-efficiency vacuum source, e.g., provided by the institution or a vacuum pump.
3. Matheson flow meter: Vacuum Meter, Cat. No. 5083R60, Thomas Scientific, Swedesboro, NJ.
4. Glo Germ™ Particles (GLo Germ Inc., Cat. No. GGP, (www.glogerm.com).
   a. Wash Glo Germ™ particles in 5 ml of ethanol at a concentration ranging from 200–400 $\times$ 10^6 particles per milliliter twice with 100% ethanol by centrifugation at 900×*g* for 10 min. If Glo Germ™ particles are obtained as powder resuspend in 5 ml of 100% ethanol before continuing.
   b. Resuspend in 100 ml of buffer (10% fetal calf serum + 1% Tween 20 + 1 mg/ml sodium azide in PBS). Note that particles can be stored in an opaque glass container at 4°C for up to one year.
   c. Filter particles through a 100 μm nylon mesh filter to create a stock suspension. Dilute with PBS (~1:20) before sorting to achieve the appropriate acquisition rate for aerosol testing, e.g., 20,000–50,000 particles per second.

Quantitative measurement of containment.

Aerosol containment system (ACS). The following protocol is designed to measure the effectiveness of an ACS on a high-pressure cell sorting flow cytometer. This protocol utilizes fluorescent particle containing aerosols, which can be trapped in a vacuum device and measured in a fluorescent microscope. In the example shown here, a BD FACSAria™ flow cytometer is described, but this protocol can be adapted to any high-speed sorter. (For additional information see the website, http://www.niaid.nih.gov/vrc/labs_flow.htm).

• The ACS must be on and functioning according to the manufacturer guidelines. Figure A1 shows an example of the aerosol flow and the locations of the vacuum gauge and monitor on a typical ACS. Using this system, the vacuum monitor is set to 20% and the vacuum gauge typically reads between 1.0 and 1.5 in. of H_2O. If it is outside of this range, the HEPA filter is replaced.

• The sheath waste tank must contain enough sodium hypochlorite to provide a final concentration of 10% when filled (1 L bleach to a final 10 L waste collected).

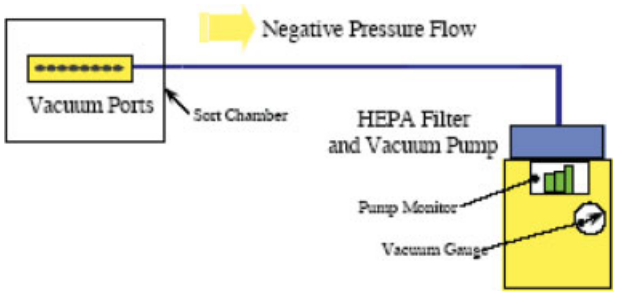


FIG. A1. Aerosol containment system. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

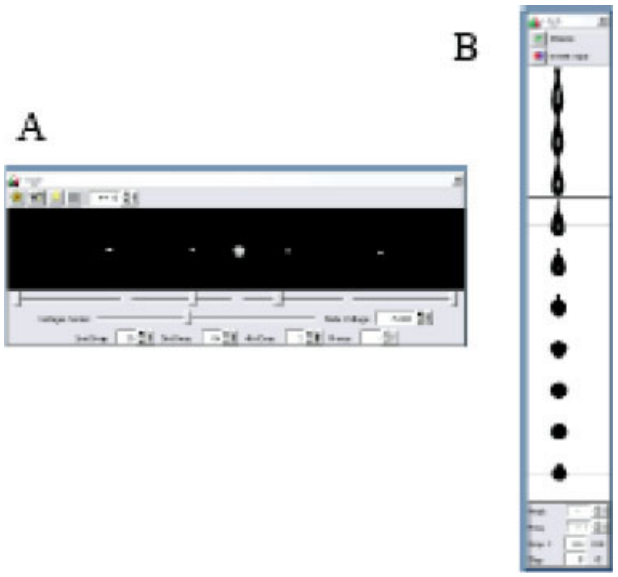


FIG. A2. Sort stream (**A**) and Accudrop camera (**B**) displays. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

• Most flow cytometers, which employ high-speed cell sorting have a digital camera system focused of the sort stream (the Accudrop™ camera is shown in this example). This camera system is used to monitor the sort stream and alerts the operator to potential sort stream disruption, which can lead to increased aerosols. Figure A2A shows the normal operating sort stream in four-way sorting mode. If the stream is disrupted the droplets form a smear in this display. In this situation, the operator can correct the sort stream and reduce aerosol contamination. Furthermore, some instruments are equipped with a droplet breakoff monitoring technology (the lines in Fig. A2B), which can detect stream drifts because of possible clogs and automatically shuts down the stream. It is highly recommended that both of these devices are employed during all sorting operations.

Measurement of containment.

• The ACS is tested under simulated worse case failure mode (active mode). In this mode the instrument is set to 70 psi and 50,000 particles/sec, with the stream hitting

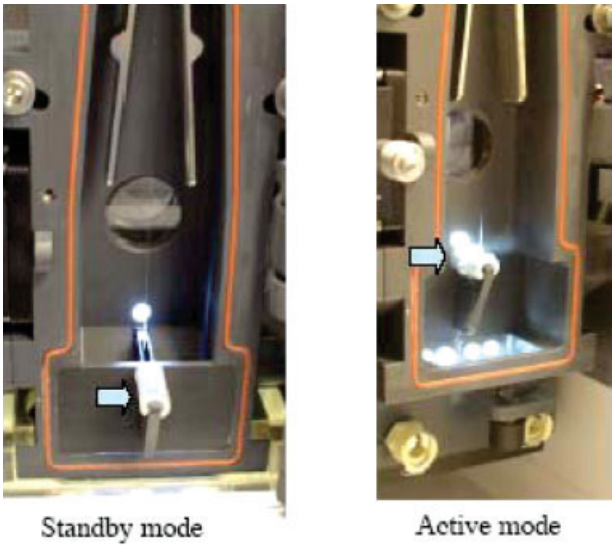


FIG. A3. Aerosol formation.[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIG. A4. AeroTech device. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the waste catcher shield to create excessive aerosol. This is accomplished by covering the waste catcher with a disposable rubber shield (Fig. A3, "standby" refers to normal stream collection; "active" refers to the failure mode).

• A glass slide is placed into the AeroTech concentrator and the parts are assembled as shown Figure A4. The vacuum pressure to the AeroTech concentrator is adjusted to a setting where between 50 and 100 particles are seen on the slide, e.g., 55 LPM (liters per minute) for the BD FACSAria. The main sort chamber is closed and the AeroTech device is placed directly on top of this chamber door as shown in Figure A5.

• The ACS is turned on according to manufacturer's instruction.

• Place GloGerm™ particles into the sample station and adjust either the particle concentration or the flow rate to achieve a particles rate of 50,000 particles per second. Note: When creating aerosols, which could contain

FIG. A5. Position of the AeroTech device. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

GloGerm™ particles, it is recommended that the operator wear a 0.2 μm particle mask.

- Begin acquiring GloGerm™ particles and allow collection for 5 min. (see an example of typical bead plots in Fig. A6). After this time, multiple areas can be measured by moving the AeroTech device to another location while continuing the particle deflection.
- Turn off vacuum to the AeroTech concentrator and remove slide. Put in a fresh slide and continue collecting aerosol with the ACS *turned off* for another 5 min. This will act as the positive control slide. Stop sample acquisition and remove shield on waste catcher.
- Examine glass slides for bright green fluorescence using a fluorescent microscope equipped with a FITC filter at 520–640 nm (Fig. A7). When a large amount of aerosol is collected (i.e. positive control slide) the Glo-Germ particles will form a pattern as directed by the holes found in the plate cover.
- Scan the entire slide using a 10× objective and count all GloGerm™ particles. The positive control slide can be used as a reference if the slide reader needs help to distinguish between fluorescent debris and actual GloGerm™ particles. Record all data.

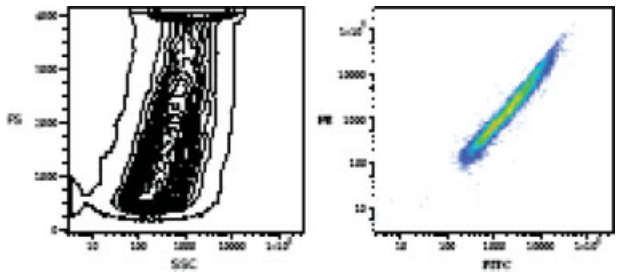


FIG. A6. Histogram of GloGerm™ particles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

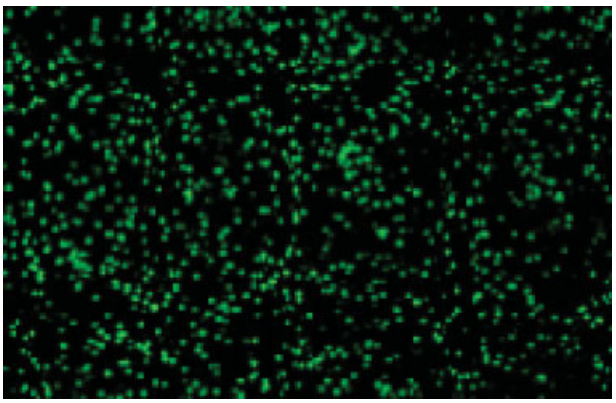


FIG. A7. GloGerm™ particles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Acceptable tolerance. All tolerance ranges as outlined by the manufacturer of the ACS must be strictly followed. Listed below are the acceptable tolerances for the measurement of containment using the GloGerm™ protocol.

- *Particles outside* = Zero tolerance, no particles on entire slide. Any positive result must be investigated, resolved and the instrument retested before proceeding with sorting potentially infectious samples.
- *Particles inside (positive control)* = Greater than 100 per slide.